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Communication

Conversion of L-Sorbosone to L-Ascorbic Acid by a NADP-Dependent Dehydrogenase in Bean and Spinach Leaf[1]

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ABSTRACT

An NADP-dependent dehydrogenase catalyzing the conversion of L-sorbosone to L-ascorbic acid has been isolated from *Phaseolus vulgaris* L. and *Spinacia oleracea* L. and partially purified. It is stable at −20°C for up to 8 months. Molecular masses, as determined by gel filtration, were 21 and 29 kilodaltons for bean and spinach enzymes, respectively. K_m for sorbosone were 12 ± 2 and 18 ± 2 millimolar and for $NADP^+$, 0.14 ± 0.05 and 1.2 ± 0.5 millimolar, for bean and spinach, respectively. Lycorine, a purported inhibitor of L-ascorbic acid biosynthesis, had no effect on the reaction.

Little is known regarding enzymic processes involved in biosynthesis of AA[3] in plants although it is firmly established through studies with specifically-labeled glucose that plants utilize a 'direct' pathway in contrast to the 'inversion' pathway found in AA-synthesizing animals. Carbon 1 of glucose is oxidized to produce the carboxyl function of AA by plants whereas carbon 6 undergoes this process in animals (9).

Basic requirements for a direct conversion of glucose to AA include oxidation of C1, an internal oxidation at C2 or C3, and epimerization of C5. The order in which these reactions occur may be other than that just given. One proposed sequence has found support through *in vivo* studies on detached *Phaseolus vulgaris* or *Spinacia oleracea* leaves that were fed solutions of [6-^{14}C]glucose or [6-^{14}C]glucosone (15). Glucosone was a more efficient precursor of AA and very little redistribution of ^{14}C occurred during its conversion. Moreover, [U-^{14}C]sorbosone was also an effective precursor of labeled AA. This sequence involves oxidation of C2 of glucose, epimerization at C5, and oxidation of C1 as the final step:

D-Glucose → D-glucosone → L-sorbosone → L-ascorbic acid

We report here the isolation from *P. vulgaris* and *S. oleracea* leaves of a NADP-dependent dehydrogenase that converts sorbosone to AA.

MATERIALS AND METHODS

Chemicals

Sorbosone and [U-^{14}C]sorbosone were generous gifts from Hoffman-LaRoche Inc. HPLC revealed a small electrochemically reactive peak close to AA in the unlabeled sample. This interferring component was removed by passing an aqueous solution of sorbosone through short columns of Dowex 50 H^+ and Dowex 1 formate exchange resins, adjusting to pH 7, and lyophilizing the effluent. GLC of the methyloxime-trimethylsilyl derivative as well as HPLC revealed only one compound, sorbosone. Tetrazolium red was purchased from Sigma Chemical Co. Lycorine (3,3a-didehydrocoran-1α,2β-diol) was provided by Professor H. Irie, Nagasaki University. Other chemicals were reagent grade or better.

Analysis of Osones

A modification of the method of Volc *et al.* (17) was used. To 0.5 mL of sample (25°C water bath) was added 0.1 mL of 1% triphenyltetrazolium chloride (tetrazolium red) in distilled water and 0.4 mL of 6 N NaOH. After exactly 5 min, 2 mL of a 1:9, v/v, mixture of acetic acid-95% ethanol was added. The mixture was spun in a vortex mixer and the absorbance measured at 480 nm against a water blank. The volume of acetic acid-ethanol mixture was varied from 2 to 15 mL, depending upon the sensitivity required. A standard curve was prepared over the range from 0 to 1 μmol of sorbosone or glucosone.

Chromatography

The purity of sorbosone was established by thin layer chromatography on cellulose in isopropyl alcohol-pyridine-acetic acid-water, 4:4:1:2, v/v (8), using alkaline silver nitrate for detection, by gas chromatography on capillary OV-1 support as the methyl oxime/trimethylsilyl ether (11) and by

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[3] Abbreviations: AA, L-ascorbic acid (L-*threo*-hexen-2-ono-1,4-lactone); glucose, D-glucose; glucosone, D-glucosone (D-*arabino*-hexos-2-ulose); sorbosone, L-sorbosone (L-*xylo*-hexos-2-ulose); Temed, *N,N,N',N'*-tetramethylenediamine.

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HPLC in 20% aqueous acetonitrile containing 3 mM (final) potassium phosphate buffer (pH 6.0) (5).

Determination of Ascorbic Acid

AA was determined by HPLC with electrochemical detection (6). Recent assays have been automated through the use of a Spectro-Physics model 8875 autosampler and model 4270 computing integrator with data acquisition module.

Assay for Sorbosone Dehydrogenase

Unless otherwise specified, enzyme (adjusted to pH 8.4) was incubated with 0.1 M bicine HCl, 1.25 mM $NADP^+$, and 25 mM sorbosone (total volume 1 mL, 30°C, pH 8.4). The reaction was terminated at 30 min (or 15 min in kinetic experiments) by addition of 0.2 mL of 30% TCA. Protein was removed by centrifugation. The supernatant was passed through Dowex 50 (H^+ form), a Sep-Pak C-18 cartridge (Millipore Corp.) and a 0.45 μm filter prior to injection of 20 μL on an HPLC column (13). When samples were prepared for autosampling, 25 μL of 3% metaphosphoric acid was added to each sample vial (0.5 mL) to preserve AA.

Spectrophotometric assays for the 310 nm absorption were measured on a Guilford-modified Beckman model DU spectrophotometer.

Preparation of Sorbosone Dehydrogenase

One-week-old bean leaves or 3-week-old spinach leaves (40–100 g) were rinsed with distilled water and spun free of excess water in a lettuce collandar. Leaves were ground in an Omnimixer (Sorvall) with two volumes (w/v) of 0.02 M Hepes buffer containing 1 mM GSH (pH 7.4).

The homogenate was filtered through six layers of cheesecloth and the filtrate centrifuged (1,000*g*, 30 min, 4°C). The supernatant was fractionated with $(NH_4)_2SO_4$. Protein that precipitated between 11.5 and 33.3 g of $(NH_4)_2SO_4$/100 mL of solution was collected by centrifugation (12,000*g*, 15 min, 4°C), redissolved in 0.02 M Hepes buffer containing 1 mM GSH (pH 7.4), and dialyzed 4 h against 3 × 100 volumes of the same buffer. This crude $(NH_4)_2SO_4$-precipitatable fraction was frozen and stored at −20°C. Prior to further purification, thawed samples were spun at 10,000*g* for 10 min to remove insoluble residues that appeared during storage.

Bean leaf preparations were loaded on a DEAE cellulose column (2 × 30 cm) with a linear gradient (100 mL) of 0 to 0.5 M NaCl in 0.02 M Tris buffer containing 1 mM GSH (pH 7.4). The activity was recovered in fractions between 0.11 and 0.24 M NaCl. Pooled, active fractions were concentrated by precipitation with 60% $(NH_4)_2SO_4$, redissolved in 0.05 M Tris acetate containing 1 mM GSH (pH 7.4), dialyzed, and loaded on a column of Sephadex G-200 (1.2 × 90 cm) that had been equilibrated with the same buffer. The activity was eluted with the same buffer or with Hepes buffer containing 1 mM GSH.

Initially, spinach leaf preparations were purified as described above but eventually a modified scheme was adopted as described here to avoid large loss of activity in the DEAE-cellulose step. The crude dialyzed $(NH_4)_2SO_4$ fraction was loaded on G-75 Sephadex (2 × 30 cm) and eluted with 0.02 M Hepes containing 1 mM GSH (pH 7.4). The activity appeared in fractions from 37 to 54 mL. These were pooled and loaded directly on Sepharose Q (1.2 × 30 cm), washed with the same buffer and eluted with a gradient of 0 to 0.4 M NaCl in the same buffer. Activity appeared between 0.19 and 0.28 M NaCl.

Activity from Sepharose Q was further purified by PAGE on a 16% native gel (7). Samples (1–5 μg protein) in 0.5 M sucrose containing 0.01% bromophenol blue were run 3 to 3.5 h at 20 mA (100 V). Following electrophoresis, gels were fixed and stained with Coomassie blue R-250. The remaining gel was sliced into horizontal 2 mm sections. Individual sections were homogenized in 0.2 M Hepes buffer, eluted, and assayed for sorbosone deydrogenase activity.

Protein was determined with Coomassie blue (2).

RESULTS AND DISCUSSION

$NADP^+$ was required for activity with both bean and spinach preparations. NAD^+ could not be substituted for $NADP^+$. Removal of O_2 from the reaction mixture by flushing with N_2 had no effect. Optimal activity for both bean and spinach enzyme was found at pH 8.3 to 8.4. When preparations were assayed for sorbosone dehydrogenase activity at this pH, 340 nm absorption due to reduction of $NADP^+$ was masked by a broad absorption with its maximum at 310 nm. This unknown peak increased during the assay. The amount of sorbosone autooxidized or converted to AA during this reaction was <0.01%. The tetrazolium red assay for sorbosone at the end of the reaction was too insensitive to detect any disappearance of sorbosone. Preincubation of sorbosone under reaction conditions for 0, 30, or 60 min prior to addition of enzyme did not affect the yield of AA making it improbable that any autooxidation product of sorbosone was the substrate. Attempts to follow $NADP^+$ reduction by spectrofluorometric assay (18) or dye reduction (10) were unsuccessful. In view of these difficulties, all determinations of enzymic activity were based on AA formation as measured by electrochemical detection with HPLC.

To characterize AA produced from sorbosone by sorbosone dehydrogenase, two experiments were run, one involving oxidation of sorbosone-derived AA with ascorbate oxidase, and one involving use of [U-^{14}C]sorbosone. In the former, aliquots from a 30 min incubation of spinach sorbosone dehydrogenase with sorbosone and $NADP^+$ (upscaled 10-fold from normal assay conditions to provide adequate sample) were passed through a column of Dowex 50 H^+ (5 mL) to stop the reaction. Aliquots were incubated at 25°C with and without ascorbate oxidase EC 1.10.3.3 (0.2 units) in 0.2 M citrate/phosphate buffer (pH 5.6) (15). After 40 min, the AA produced in the dehydrogenase reaction was completely oxidized as compared to only 50% oxidation in controls. HPLC with electrochemical detection is specific for the reduced form of AA. Ascorbate oxidase is highly specific for compounds that contain the enediol lactone configuration as present in carbons 1 through 4 of AA.

In the second experiment on characterization of the enzymatic product, [U-^{14}C]sorbosone was used as substrate. A simultaneous run with unlabeled substrate provided material

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Table I. *Partial Purification of L-Sorbosone Dehydrogenase from Spinach (305 g)*

Fraction	Protein	Activity	Recovery	Specific Activity
	mg	*pkats*	*%*	*pkats/mg protein*
45–55% $(NH_4)_2SO_4$	350	420	100	1.2
Sephadex G-75	100	348	83	3.5
Sepharose Q	12.5	86	20	6.9
PAGE (Sections 15–18)	0.17	8.5	2.1	50[a]
Section 15	0.08	0.4	0.1	5
Section 16	0.05	3.6	0.9	72
Section 17	0.02	3.0	0.7	150
Section 18	0.02	1.5	0.4	75

[a] Total pkats/total mg protein in PAGE sections 15 through 18. Protein in gel sections was estimated spectrophotometrically by its 260/280 nm absorption due to the low values encountered.

for assay by HPLC. After 30 min of incubation, the reactions were terminated. The unlabeled run contained 5.4 μg of AA as assayed by HPLC. The labeled run was diluted with unlabeled AA as carrier and subjected to ion exchange chromatography to recover AA as a crystalline solid which was converted to its 5,6-monoisopropylidene derivative and recrystallized (15). Based on the recovery of ^{14}C in the derivative, 5.5 μg of AA was formed, comparable with the value obtained by HPLC. These experiments provide additional evidence for authenticity of the product as AA beyond that already established by use of HPLC as an assay (6).

Initially, the enzyme used in this study was isolated from bean leaf but undependable yield prompted a change to spinach, a reliable year-around source. To protect the quality of the costly HPLC column, raw extracts were not normally assayed for sorbosone dehydrogenase. In two instances, however, raw spinach extract after desalting through a Sephadex G-75 column was assayed to show that 9 to 18% of the total dehydrogenase activity was recovered in the 45 to 55% $(NH_4)_2SO_4$ fraction which was assigned a value of 100%. In 29 preparations, the activity at this stage of purification was 2.8 ± 1.6 pkats/g of fresh tissue. A representative example of purification from spinach is given in Table I.

Both bean and spinach enzyme, purified through the gel filtration step, could be stored for as long as 8 months at −20°C. Attempts to include a DEAE-cellulose ion exchange step following recovery of the $(NH_4)_2SO_4$ precipitate resulted in considerable loss of activity during salt elution from the column. Vertical native PAGE gels revealed 4 to 5 protein components in the sorbosone dehydrogenase activity that was recovered from Sepharose Q. This activity which was distributed over the two most rapidly migrating PAGE bands could not be further resolved. When a typical gel was sectioned into 2 mm portions as in Table I, the activity appeared in 4 sections (No. 15–18) whose average specific activity was 50 pkats/mg of protein. The highest specific activity, 150 pkats/mg, appeared in section 17.

Approximate molecular masses of bean and spinach sorbosone dehydrogenase after partial purification of the 45 to 55% $(NH_4)_2SO_4$ fraction through DEAE cellulose and gel filtration on Sephadex G-150 (an earlier procedure that was later replaced by the Sephadex G-75 step) were 21 ± 1 and 29 ± 2 kD, respectively.

Sorbosone dehydrogenase that had been purified through the Sephadex G-200 step (bean) or the Sepharose Q step (spinach) was used to obtain kinetic constants. AA production was linear for the first 20 min of the reaction. Rates were determined in duplicate at five concentrations of sorbosone or $NADP^+$ and the data analyzed by Cleland's hyperbolic computer program (3). At 30°C and 1 mM $NADP^+$, the apparent K_ms for sorbosone were 12 ± 2 and 18 ± 2 mM, while at 50 mM sorbosone, the corresponding K_ms for $NADP^+$ were 0.14 ± 0.05 and 1.2 ± 0.5 mM for bean and spinach enzymes, respectively.

Lycorine, an alkaloid present in amaryllidaceous plants, and certain of its derivatives are reported to be inhibitors of AA biosynthesis in plants (1, 4). When lycorine was included in the sorbosone dehydrogenase assay over a concentration range of 0.1 to 100 μM, no inhibition was detected. In other *in vivo* experiments, lycorine was supplied through the freshly cut petiole to detached 20-d-old spinach leaves for 12 h in the presence (30 $\mu E/m^2 \cdot s$) or absence of light at concentrations ranging from 0.1 μM to 1 mM. No significant change in AA content (627 ± 61 g AA/g fresh weight of leaf) was detected as compared with water controls (591 ± 69 g AA/g fresh weight) but a concentration of lycorine greater than 0.1 mM caused visible shrinking of petiolar tissue and eventual wilting. A similar result was obtained when detached leaflets from either young or mature vines of *Parthenocissus quinquefolia* (Virginia creeper) were supplied with lycorine in analogous experiments. Spinach and Virginia creeper leaves metabolize AA by uniquely different pathways (14) although it is likely that AA biosynthesis follows a process common to both (9). Given the highly selective, specific and sensitive method of AA analysis used in this study, it is unlikely that lycorine has an inhibitory effect on sorbosone dehydrogenase-catalyzed synthesis of AA although it may have a more general cytotoxic effect on translocation and respiration.

Conversion of sorbosone to AA involves oxidation of carbon 1 and formation of a 1,4-lactone. In view of the $NADP^+$ requirement, this conversion is most suitably explained as a dehydrogenation followed by lactonization. K_ms for sorbosone, and to a lesser degree for $NADP^+$, were higher than expected which may be explained by a two-step process. Alternatively, the functional substrate might be a minor isomeric form of sorbosone, possibly an enolic structure such as

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that described for glucosone (8), which upon oxidation at carbon 1 will lactonize spontaneously to form an isomeric AA. Assuming that such a structure is present as a component of sorbosone under the weakly alkaline conditions that prevail during sorbosone to AA conversion, then a single enzymatic step will suffice. In this case, saturation will require a very high concentration of sorbosone whereas $NADP^+$ will reach saturation at a more reasonable concentration.

In the first instance, the intermediate would be 2-keto-L-gulonate (L-*xylo*-2-hexulonate) as in animal AA biosynthesis. When this compound was tested as substrate over a range of 0.05 to 68 mM with sorbosone dehydrogenase, no AA was formed. One possible explanation can be found in metabolite transfer via enzyme-enzyme complexes in which a tightly bound intermediate is involved (12, 16). L-Idonate (10 mM), L-galactonate (50 mM), and L-galactono-1,4-lactone (50 mM) were also tested and gave negative results. At this point, the study has failed to reveal involvement of two enzymes in conversion of sorbosone to AA although the two unresolved PAGE bands make this a possibility.

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